Interactions of heterologous DNA with polyomavirus major structural protein, VP1

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Abstract 'Empty' polyomavirus pseudocapsids, self-assembled from the major structural protein VP1, bind DNA non-specifically and can deliver it into the nuclei of mammalian cells for expression [Forstová et al. (1995) Hum. Gene Ther. 6, 297–306]. Formation of suitable VP1-DNA complexes appears to be the limiting step in this route of gene delivery. Here, the character of VP1-DNA interactions has been studied in detail. Electron microscopy revealed that VP1 pseudocapsids can create in vitro at least two types of interactions with double-stranded DNA: (i) highly stable complexes, requiring free DNA ends, where the DNA is partially encapsidated; and, (ii) weaker interactions of pseudocapsids with internal parts of the DNA chain.

Key words: Capsid-like particle; DNA-protein interaction; DNA encapsidation; Gene therapy; Polyoma virus

1. Introduction

Polyoma virus, the mouse member of the papova family, is a small non-enveloped tumorigenic virus with a circular genomic DNA (5.3 kbp) that encodes six proteins, three early antigens and three late structural proteins, VP1, VP2 and VP3. The late proteins, together with viral DNA and cellular histones, H2A, H2B, H3 and H4, are assembled in the host cell nuclei into virions. The icosahedral capsids of the virions are made up of 72 capsomeres. Neither VP2 nor VP3 are required for the capsid-like architecture. VP1 alone can self-assemble into empty capsids and make the three non-equivalent types of interactions to produce 60 pentavalent and 12 hexavalent pentameric capsomeres [2–4]. The position of the two minor structural proteins in virions has been deduced from X-ray diffraction data, where the electron density map of the inside of the virion displayed 72 prongs of density extending from the core into the axial cavities of the VP1 pentamers. These prongs were identified with the C-terminal part of either VP2 or VP3 molecules [5]. Amino acids 140–181 in VP3 (also present in VP2) have been proposed as being responsible for the interaction of the minor structural proteins with capsomeres of VP1 [6]. The nucleocore of the virion is formed by genomic circular double-stranded (ds) DNA in a complex with host cell histones and VP1. VP2 and VP3 may function to guide the assembly of the capsid onto the nucleohistone core [5]. VP1 has a non-specific DNA binding activity, located in several amino acids within the amino acid N-terminus [7,8]. The presence of 'naked' DNA has been reported to inhibit in vitro capsomere self-assembly into capsid-like structures [8]. In contrast to a member of the same virus family, SV40, where the minor structural proteins have non-specific DNA binding activity [9], there is no DNA binding activity connected with VP2 and VP3 of mouse polyomavirus [7].

We previously demonstrated that mouse polyomavirus pseudocapsids, self-assembled from VP1 produced in insect cells, can interact with heterologous DNA in vitro and deliver it into the nuclei of mammalian cells for expression [1,10]. Preparation of suitable DNA complexes was an important step affecting the efficiency of this route of gene delivery. We found that short fragments (up to 2 kbp) of DNA in complexes with VP1 pseudocapsids could be entirely protected from DNase I cleavage. The amount of protected DNA was extremely variable (from 2 to 30% of input DNA in individual experiments) [1]. The analysis of pseudocapsid preparations from insect cells revealed that a variable proportion of pseudocapsids encapsidated DNA together with cellular DNA-binding proteins already inside insect cells [11,12]. Here we analyse the character of the in vitro interactions between heterologous DNA and 'empty' VP1 pseudocapsids using gel retardation electrophoresis and electron microscopy. Elucidating the structures of the complexes that can be formed between DNA and VP1 pseudocapsids, and the factors responsible for variability of packaging efficiencies, is an important topic to be addressed in future gene therapy experiments using the pseudocapsids.

2. Materials and methods

2.1. Cells and viruses

Spodoptera frugiperda cells (Sf9) were grown as monolayer cultures at 27°C in TNF-FH medium containing 10% foetal calf serum (FCS) as described by Hink [13]. Recombinant baculovirus containing the polyomavirus VP1 gene was used for the infection of Sf9 cells [10,14]. Infected cells were harvested 72 h post infection.

2.2. Isolation of polyomavirus capsid-like particles from insect cells

VP1 capsid-like particles were isolated from infected Sf9 cells by CsCl and sucrose gradient centrifugation as described previously [1]. Fractions containing particles were concentrated by pelleting through a 10% sucrose cushion and resuspending in buffer B (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 0.01 mM $CaCl_2$).

2.3. Preparation of VP1 capsomeres

VP1 pentameric capsomeres were obtained by disassembly of puri-

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Abbreviations: VP1, major capsid protein; VP2,3, minor capsid proteins; EM, electron microscopy; BAC, benzyldimethylalkylammonium chloride

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fied VP1 capsid-like particles in the presence of 3–10 mM DTT and 1 mM EGTA, as described [15].

2.4. DNAs

The 6238 bp plasmid, pCDNA3-CAT [1], and the 2015 bp plasmid, pHC624 [16], were used as supercoiled or open circle forms, or as linear DNA, linearised with appropriate restriction endonucleases. Cytoplasmic linear ds DNA plasmids, pGKLs 1 and 2, of *Kluyveromyces lactis*, with protein bound on 5′ ends [17], were treated with proteinase K before the complex formation experiment.

2.5. Protein analysis

Protein concentrations in pseudocapsid preparations were estimated according to Lowry et al. [18]. Purity of the pseudocapsid preparations were tested on SDS-12.5% PAGE gel and visualised by Coomassie blue and Western blot analysis using monoclonal αpyVP1-A antibody [10,19].

2.6. Complex formations

Complexes of VP1 structures with DNA were performed by incubation of the mixture of DNA with 5-fold molar excess of pseudocapsids in buffer B for 1 h at 37°C, followed by exposure to osmotic shock, according to a previously described procedure [20].

2.7. Electron microscopy (EM)

Polyomavirus VP1 particles and natural virions were visualised by negative staining. Parlodion-carbon coated grids, activated with glow discharge [21] were floated on the top of a 15 μl drop of sample, washed in distilled water and transferred on a drop of 1% phosphotungstic acid (pH 7.0), left for 1 min and dried. Complexes of VP1 and DNA were prepared for EM using modified aqueous or BAC spreading techniques as described elsewhere [22]. In some cases the DNA-pseudocapsid complexes were fixed in 0.15% (v/v) glutaraldehyde [23]. Electron micrographs were recorded in a JEM 1200EX electron microscope (JEOL) operating at 60 kV. Length measurements were performed with an Olivetti PD digitiser linked to a Golem AT 386 PC system.

2.8. Gel retardation assays

Complexes of plasmid DNA and VP1 structures were separated by electrophoresis on an 0.8% agarose gel in 0.5×TBE buffer [16].

2.9. Protection of DNA against nucleases in complexes with VP1

Complexes of DNA and VP1 were incubated in the presence of 10 mM $MgCl_2$ with DNase I (100 μg/ml) at 37°C for 1 h. DNase I activity was stopped by the addition of EDTA (final concentration 100 mM) and SDS (0.5%). Samples were treated with preincubated pronase E (250 μg/ml) 1 h at 37°C, followed by phenol-chloroform extraction and ethanol precipitation.

3. Results

3.1. VP1 pseudocapsids from insect cells

In CsCl gradient centrifugation, two main VP1-containing bands were obtained, one with buoyant density (ρ = 1.283–1.294 g/cm^3), corresponded to the density of 'empty capsids' and the other (heavy pseudocapsids), with buoyant density (ρ = 1.315–1.348 g/cm^3), corresponding to that of infectious polyoma virions. The former, designated pseudocapsids, were further purified in sucrose gradients and their diameter was calculated as 41 ± 4.2 nm (from negative staining EM). Both electron microscopy and SDS gel electrophoresis analysis confirmed the homogeneity of the pseudocapsid preparation (not shown).

3.2. In vitro interactions of DNA and VP1 particles

3.2.1. Gel retardation assays. Both VP1 pseudocapsids

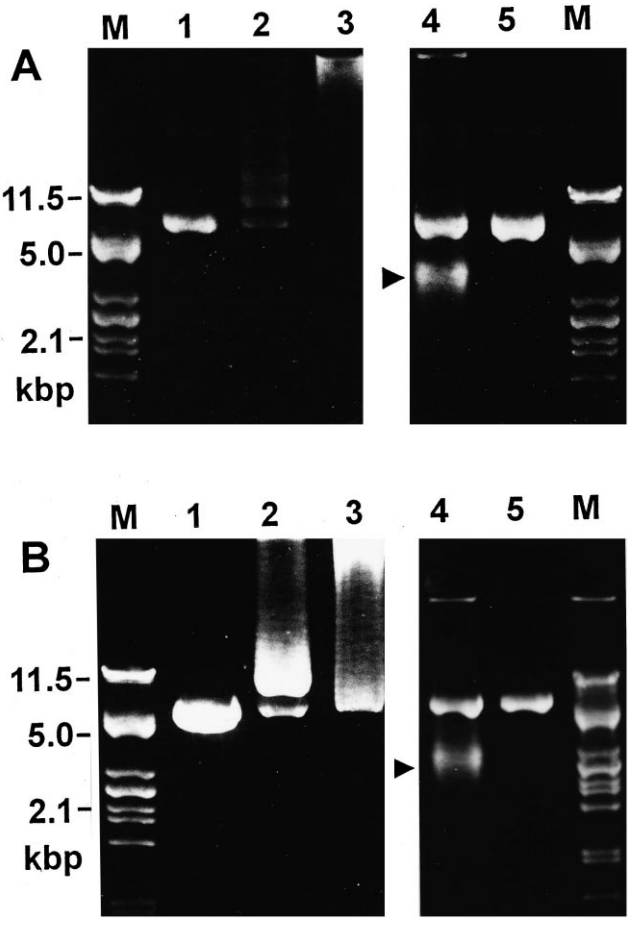

Fig. 1. Gel retardation assay. (A) Linearised and (B) circular 6.2 kbp plasmid DNA complexed with 'empty' pseudocapsids (tracks 2), with pentameric capsomeres (tracks 3), and with 'heavy' pseudocapsids (tracks 4). Tracks 5 contain plasmid DNA with wild type polyoma virions. Complexes were run on an 0.8% agarose gel. M: molecular weight markers. Arrowheads represent the ethidium bromide stained band of 'heavy' pseudocapsids.

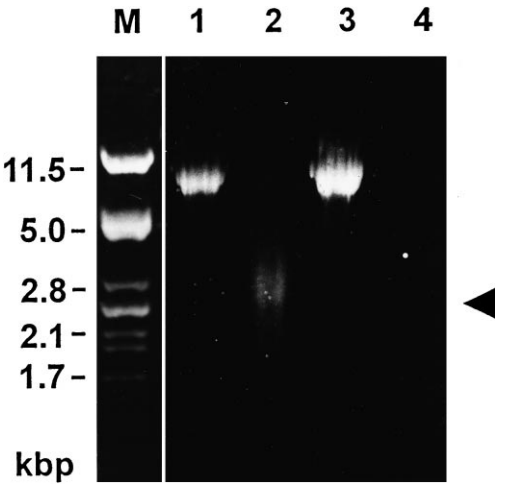

Fig. 2. Treatment of complexes with DNase I. Agarose gel electrophoreses of DNA complexed with VP1 structures in vitro. Linear DNA (6.2 kbp) mixed with 'empty' pseudocapsids (tracks 1 and 2) or with capsomeres (tracks 3 and 4). Tracks 2 and 4: complexes treated with DNase I, deproteinated and analyzed by agarose gel electrophoresis; tracks 1 and 3: DNase I treatment omitted. The arrowhead indicates the diffuse band of DNA fragments protected by pseudocapsids. Bands are identified by staining with ethidium bromide.

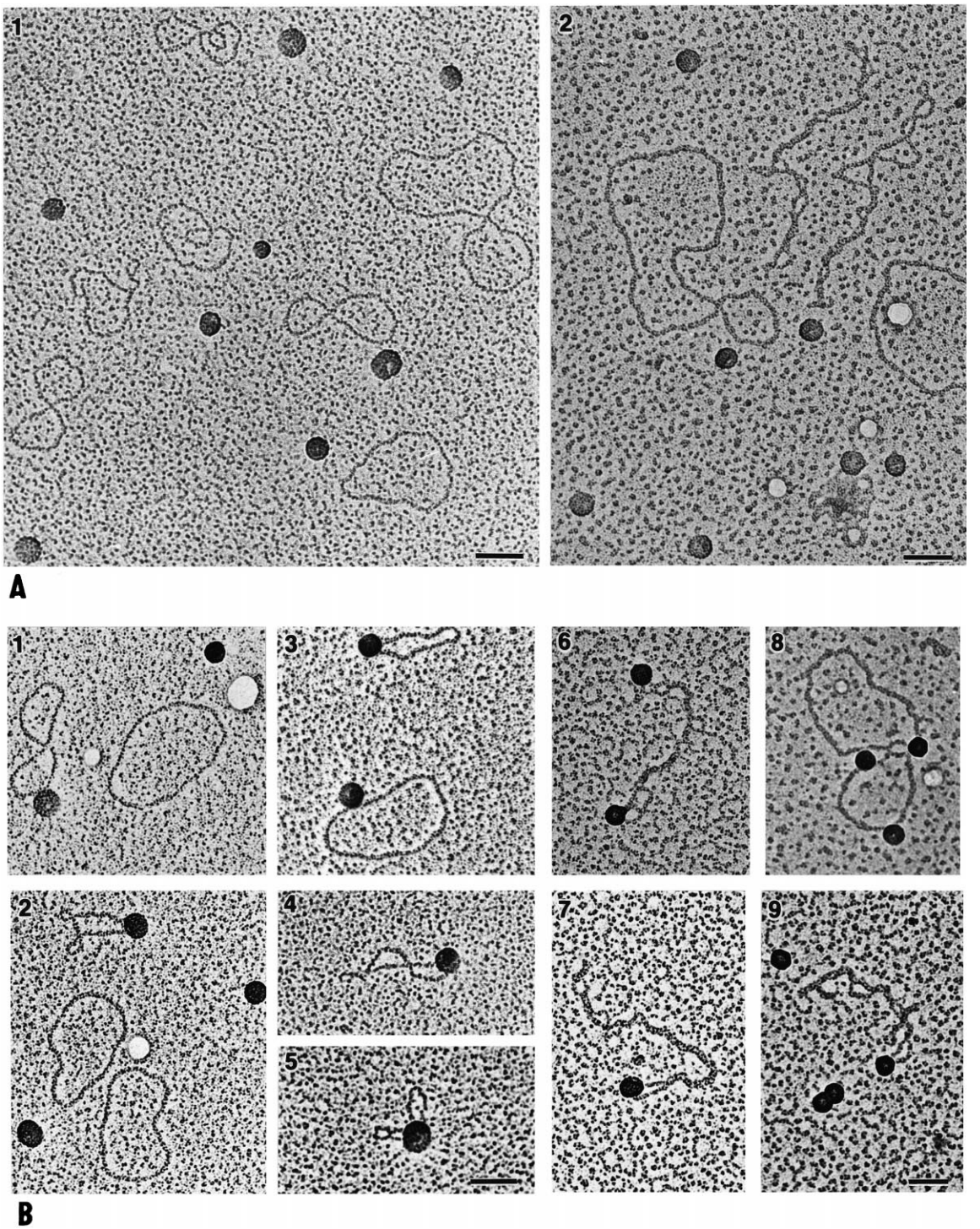

Fig. 3. Electron micrographs of interactions observed between VP1 pseudocapsids and circular heterologous DNAs. A: Typical fields (90% of those analysed) where no evidence of interaction was observed. B: Selected DNA molecules found in contact with pseudocapsids (10% of fields). Bacterial plasmids pHC624, 2 kbp long (A1, B1–5), and pCDNA3-CAT, 6.2 kbp long (A2, B6–9), were used for complex formation with pseudocapsids. Aqueous spreading technique, bars = 0.1 μm.

and VP1 pentameric capsomers (see Section 2) interact with circular supercoiled and linearised DNAs and decreased their mobilities, as shown respectively in Fig. 1A,B. The interaction of capsomeres (tracks 3) mediated a more dramatic shift, essentially preventing most of the DNA from migrating into the gel, while DNA-pseudocapsid complexes formed distinct bands migrating between the initiation point and the position of free DNA (tracks 2). These data suggest that VP1 capsomeres form more irregular and voluminous aggregates with DNA and/or saturate the negative charges on the DNA. Circular DNA-pseudocapsid complexes formed ladders of progressively shifted bands, while one abundant major band and several less mobile minor bands could be observed when linear DNA was used for the interactions with pseudocapsids.

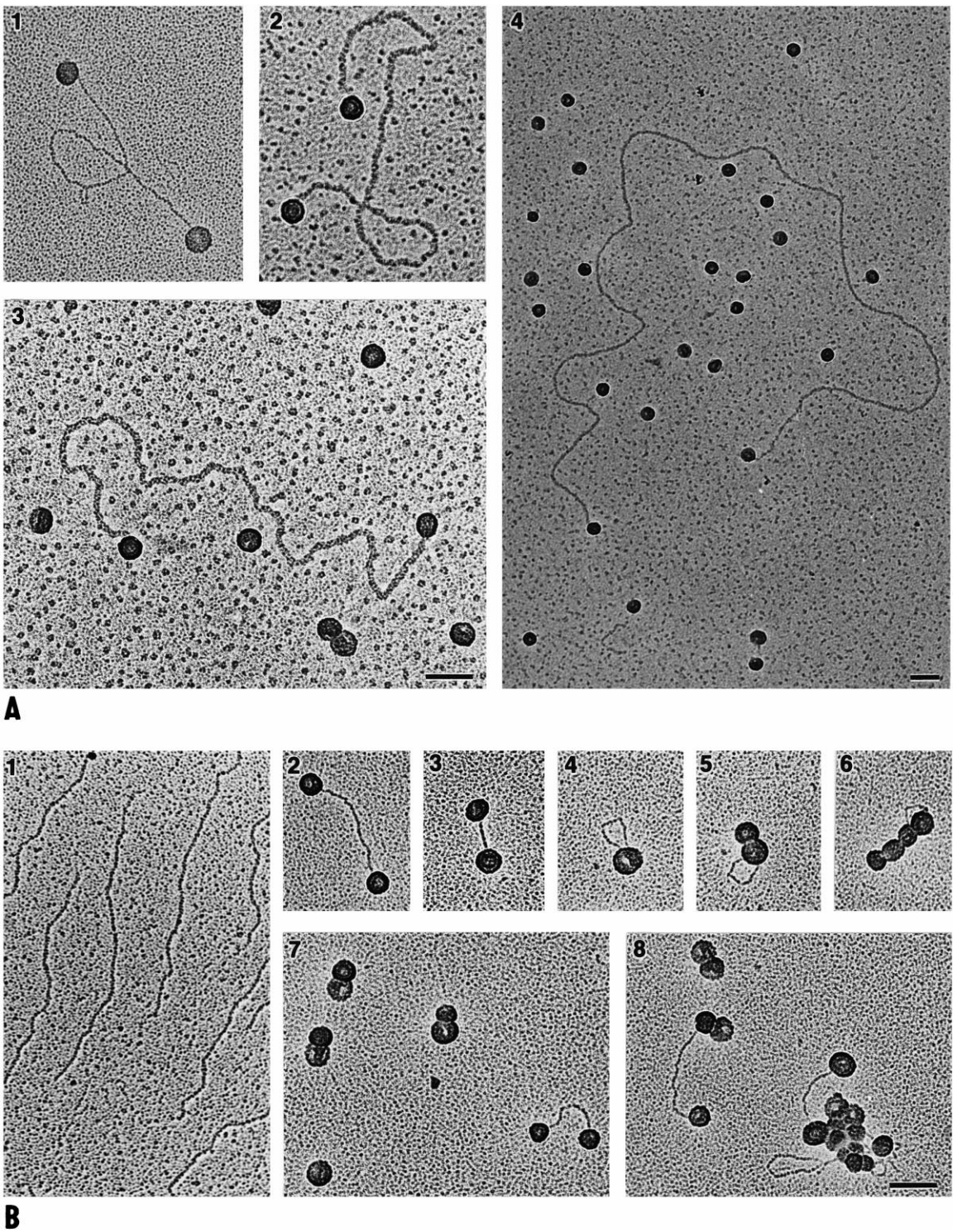

Fig. 4. Electron micrographs of interactions of VP1 pseudocapsids with linear DNAs. A: Interactions with: (panels 1,2) plasmid pCDNA3-CAT (6.2 kbp) linearised with *Bgl*II; (panels 3,4): a linearised concatemer of pCDNA3-CAT. Bar = 0.1 μm. B: (panel 1) pHC624 DNA (2 kbp) linearised with *Bam*HI, and (panels 2–8) complexed with pseudocapsids. BAC spreading technique, bar = 0.1 μm.

The heavy particle fractions, as well as polyoma virions, did not retard the migration of exogenous DNA (tracks 4 and 5, respectively). Heavy VP1 particles, in contrast to polyoma virions, gave a band that could be stained by ethidium bromide.

3.3. Protection of DNAs in VP1 complexes against DNase I degradation

To establish the level of DNA protection provided by VP1 pseudocapsids or pentameric capsomeres, DNA-VP1 complexes were digested with an excess of DNase I in the presence

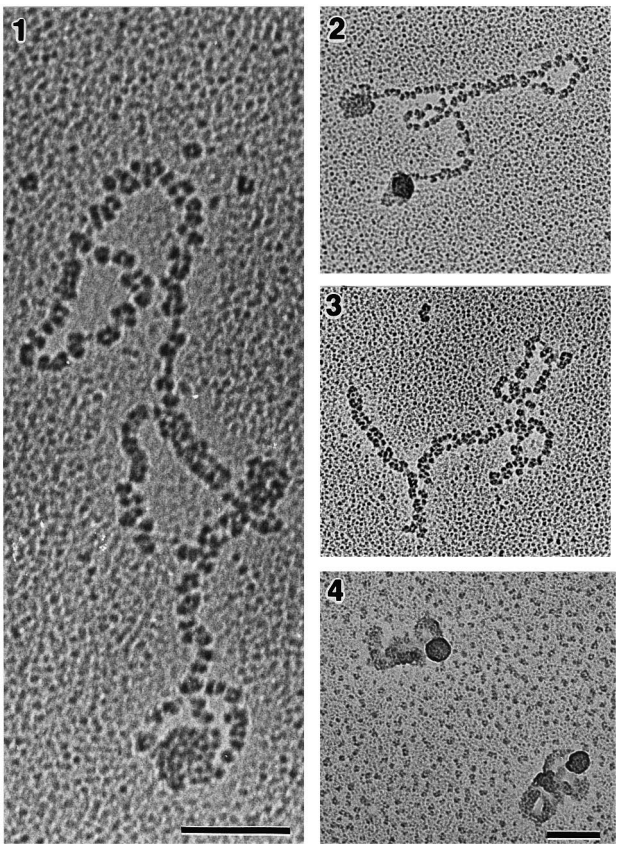

Fig. 5. Electron micrograph of complexes of DNA and VP1 capsomeres. (Panels 1–3) BAC technique, (panel 4) aqueous technique Bars = 0.1 μm.

of Mg^{2+} and the length of the protected product was estimated by agarose gel electrophoresis. Using a linearised 6.2 kbp plasmid DNA, a diffuse band of approximately 2.5 kbp in size was found to be protected from digestion by VP1 pseudocapsids (Fig. 2, track 2). When supercoiled 6.2 kbp plasmid DNA was used a similar diffuse band of protected DNA was observed, (not shown). It was difficult to distinguish between part of supercoiled DNA being encapsidated (and protected) by the pseudocapsids, and the protected DNA fragments arising from a subpopulation of linearised molecules, possibly formed during the DNase I treatment. We did not observe any protection of DNA in complexes with VP1 pentameric capsomeres (Fig. 3, track 4).

3.4. Electron microscopy analysis of DNA-VP1 complexes

For a better understanding of the nature of VP1-DNA interactions, an EM analysis was performed. Samples were prepared for EM analysis using two spreading techniques. Both modified aqueous and BAC spreading techniques gave comparable results.

When the specimens for EM were prepared from intact circular supercoiled or open circular forms of plasmid DNA and VP1 pseudocapsids, few or no interactions were observed by either aqueous or BAC methods in most fields (Fig. 3A). In some experiments about 10–20% of circular DNA molecules were found in contact with pseudocapsids (Fig. 3B, panels 1–9), and the shortening of some open DNA circles in these complexes could be observed, suggesting partial encapsidation (Fig. 3B, panels 2–4). On the other hand, linearised 6.2 kbp plasmid DNA (Fig. 4A, panels 1, 2) or its multimers (Fig. 4A, panels 3, 4) showed a high degree of interaction with pseudocapsids. In these cases pseudocapsids were mainly located at both ends of the DNA chain, forming a 'skipping rope'-like arrangement (in more than 90% of observed DNA molecules). Two pseudocapsids bound to the linear DNA (one to each end) shortened monomer lengths to 1.13 ± 0.20 μm (that is, 3.71 ± 0.66 kbp; calculated from 95 DNA molecules) in comparison to free 6.2 kbp DNA at 1.88 ± 0.096 μm (6.24 ± 0.32 kbp) long. The length of DNA molecules with pseudocapsids bound to only one end (eight such complexes were found) was on average 1.51 μm (or 4.95 kbp). These results suggest that VP1 pseudocapsids encapsidate on average 0.37 μm (1.24 kbp) of ds DNA. Encapsidation of DNA by pseudocapsids was more obvious when 2 kbp long linearised DNA molecules were used for complex formation (Fig. 4B). DNA in such complexes is shortened (Fig. 4B, panels 2–6) in comparison with free DNA (Fig. 4, panel 1), and often two pseudocapsids containing completely encapsidated DNA were observed (Fig. 4B, panels 7, 8). Larger aggregates of pseudocapsids and DNA were also present (Fig. 4B, panels 6, 8). We also observed that not only pseudocapsid structures, with 72 pentamers, but also small particles consisting of 24 pentamers, bind free DNA ends efficiently (not shown).

VP1 pentameric capsomeres exhibited very high affinity to DNA molecules, decorating them along their entire length, causing shortening, particularly visible when using the aqueous technique (probably by bending or forming small loops, not distinguished by EM) (Fig. 5).

To assess the requirements for DNA encapsidation, different substrates were exposed to pseudocapsids and prospective complexes analysed by EM. Encapsidation of linear DNA at both ends was observed when 3′ or 5′ cohesive or blunt ends (created by restriction enzymes) were used. Dephosphorylation of the 5′ end did not influence pseudocapsid binding. Amino acid residues, however, covalently bound to 5′ ends of DNA (cytoplasmic linear ds DNA plasmids, pGKLs, of *K. lactis*, with protein bound on the 5′ ends, treated with proteinase K) entirely prevented interactions with pseudocapsids (not shown).

4. Discussion

VP1, the major polyomavirus structural protein, produced in insect cells from a recombinant baculovirus, can assemble in cell nuclei into capsid-like structures [3,10]. Empty particles, which we call pseudocapsids, can interact with DNA in vitro, and also in the nuclei of insect cells infected with recombinant baculovirus [1,11,12]. VP1 produced in *Escherichia coli* has been found to bind DNA non-specifically, using several N-terminal amino acids [7,8]. Produced as pentameric capsomeres, VP1 from *E. coli* formed 'ribbon-like arrays of closely opposed capsomers' with DNA; the presence of DNA inhibited in vitro capsomere self-assembly into capsid-like structures; dithiothreitol caused significant inhibition of DNA binding [8].

We earlier demonstrated that empty pseudocapsids can form complexes with heterologous DNA fragments and deliver them into the nuclei of mammalian cells for the expression [1]. In order to optimise the delivery protocol, we have now studied the nature of the interactions between DNA and VP1 structures.

Gel retardation assays (see Fig. 1A,B) suggested that complexes of pseudocapsids with circular and linearised DNAs might have different characters. The 'ladder' of progressively shifted bands observed in gel retardation assays of pseudocapsids with circular DNA (Fig. 1B, track 2) could represent increasing numbers of plasmid DNA molecules bound to one pseudocapsid, or alternatively, increasing numbers of pseudocapsids bound to one DNA molecule. Linear DNA in complex with VP1 pseudocapsids formed preferential complexes represented by one abundant band (Fig. 1A, track 2). 'Heavy' pseudocapsids which had encapsidated DNA within the insect cells, and infectious polyoma virions, did not shift the positions of exogenous DNA. The data suggest that DNA encapsidated in vivo may change the conformation of the VP1 protein in such a way that the DNA binding N-terminal arms of the pentameric capsomeres (which form the capsid) are no longer available for interaction with exogenous DNA.

A surprising aspect of the interactions between DNA and pseudocapsids revealed by EM analysis, is that VP1 pseudocapsids can create in vitro at least two types of interactions with double-stranded (ds) DNA: (i) highly stable complexes, requiring free DNA ends, where the DNA is partially encapsidated; and, (ii) weaker interactions of pseudocapsids with internal parts of the DNA chain which can be interrupted easily by EM spreading technique. Whilst both linear and supercoiled DNA or open circular forms could be shown effectively to form complexes with pseudocapsids by gel retardation assays (and also sucrose gradient centrifugation, data not shown), 80–90% of circular DNA molecules were found to be free of pseudocapsids when mixed with a 5-fold excess of the latter, and complexes examined by EM. Looking at many EM fields we could only find 15% of circular DNA molecules that contacted one, or (rarely), more pseudocapsids. The discrepancy between the various detection methods can probably be explained by the mechanical forces or electrostatic and other interactions to which complexes are exposed during the preparation of EM samples. Interruptions of some DNA-protein complexes during spreading of EM samples were observed previously [24]. Stabilisation of complexes by glutaraldehyde fixation [25] did not, in our hands, increase the frequency of contacts. From observations of some interactions with internal regions of DNA, and from the measurement of DNA lengths, it is obvious that circular molecules can be partially encapsidated by pseudocapsids possibly via loop formation (see Fig. 3B, panels 3–5). This interaction seems to be much weaker, when compared with encapsidation via DNA ends (Fig. 4), allowing DNA to be easily released from such complexes. It is a question whether preferential appearence of pseudocapsids on free DNA ends (with partial encapsidation) is due to higher affinity between the species, or whether partially encapsidated circular DNA can be more easily removed on being exposed to double mechanical strain.

VP1, as isolated from insect cells, is present in the form of capsid-like structures. These can be disassembled into capsomeres by removal of calcium ions by EGTA in the presence of the disulphide reducing agent, DTT, as described [14]. Interestingly, these capsomeres interact efficiently with DNA, even in 10 mM dithiothreitol, and in contrast to data obtained by Southwestern assays [8]. Electron microscopy, using the protein-free BAC spreading technique, showed 'stretched' complexes with capsomeres bound along the entire DNA chain (Fig. 5). With an aqueous variant of the Kleinschmidt spreading technique, more compact structures could be seen in which DNA was efficiently bent by VP1. Partially assembled pseudocapsids were observed in some complexes, usually at the ends of the DNA chains. DNA in complexes with capsomeres was sensitive to DNase I treatment, even when high excesses of VP1 pentamers were used for complex formation.

Preparations of virions from wild type polyomavirus infected mouse cells, as earlier shown, also contain pseudovirions, that is, particles, which harbour fragments of host chromosomal DNA, with average lengths around 2–2.5 kbp [26]. This length corresponds to that protected in in vitro formed DNA-VP1 pseudocapsid complexes (also in in vitro prepared complexes formed with natural polyomavirus empty capsids) [20] and represents apparently the length of 'naked' DNA which can be accommodated by empty particles. On electron micrographs, the length of DNA encapsidated by each pseudocapsid was estimated to be about 1.2kbp. This apparent size discrepancy may reflect mechanical strain during EM spreading.

Only a few proteins or protein complexes that bind selectively to DNA ends have been described. One well studied example is the heterodimeric protein Ku (consisting of 70 and 80 kDa protein subunits), the DNA binding component of a DNA-dependent protein kinase (DNA-PK). Ku, similar to pseudocapsids, interacts specifically with free ends of linear double-stranded DNA and protects them whether they are cohesive or blunt; its binding is also independent of a terminal 5′-phosphate group. Ku requires a free end for initial interaction with DNA, then it can translocate along the DNA molecule. Ku also binds to ds DNA with nicks and gaps and to duplexed DNA ending with hairpin loops [27–29]. Because the protein kinase activity is DNA-dependent, binding is thought to induce a conformational change in the kinase subunit that activates or exposes the kinase catalytic domain [30]. VP1 pseudocapsid, after interaction with one DNA end, also may undergo a conformation change, which prevents further DNA binding, since on EM we have never seen two or more DNA chains attached to one pseudocapsid, even when high excesses of linear DNA molecules were used for complex formation. From the measurement of DNA lengths in DNA-pseudocapsid complexes it was obvious that many particles were not fully saturated by the length of encapsidated DNA.

Understanding the nature of the binding between VP1 pseudocapsid and DNA, and its particular affinity for free DNA ends, now allows us to design suitable DNA molecules for more effective in vitro encapsidation and gene therapy experiments. A recent interesting finding is that expression of a reporter gene, delivered into cell culture via pseudocapsids, can be substantially increased if a copy of it (with regulatory sequences) was engineered onto each end of a linear spacer DNA molecule and, after encapsidation, the two pseudocapsids at each end were separated by specific cleavage of the spacer DNA (unpublished results). This experiment suggests that for efficient cell entry, the preference is for only one pseudocapsid particle.

Encapsidation of naked ds DNA from free ends may be irrelevant for morphogenesis of virions where 5.3 kbp genomic DNA is present in circular supercoiled form and complexed with cellular histones. Strong affinity of pseudocapsids to DNA free ends with the ability to pull DNA in, can be an artefact of the in vitro system or a relic and redundant prop-

erty of assembled VP1 protein. On the other hand, it is apparent that VP1 interactions with DNA play a role in viral nucleocore condensation and it cannot be ruled out that the interactions of VP1 capsid-like structures with transiently formed free ends of DNA of either host cell or viral origin might be involved in virion assembly processes.

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